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Subcellular Localization of the Inducible *Chlorella* HUP1 Monosaccharide-H^+ Symporter and Cloning of a Co-Induced Galactose-H^+ Symporter[1]

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The unicellular green alga *Chlorella kessleri* can induce monosaccharide-H^+ symport catalyzing the energy-dependent transport of D-glucose (D-Glc) and several other pentoses and hexoses across the plasmalemma. The gene coding for the inducible HUP1 monosaccharide-H^+ symporter has been cloned and the protein has been characterized previously. The data presented in this paper demonstrate that the presence of the *HUP1* gene product alone is not sufficient to cover the broad substrate specificity of monosaccharide transport in induced *Chlorella* cells. Two other *HUP* genes are shown to be co-induced in *Chlorella* in response to D-Glc in the medium. The cloning of *HUP2* and *HUP3* cDNA and genomic sequences is described, both being very homologous to *HUP1*. Modification of the 5′ untranslated sequences of full-length cDNA clones of *HUP2* and *HUP3* allowed the functional expression of both transporters in *Schizosaccharomyces pombe*. HUP2 was shown to be a galactose-H^+ symporter, whereas the substrate specificity of the *HUP3* gene product is very similar to that of the HUP1 protein. However, *HUP3* does not seem to be induced to high levels in Glc-treated *Chlorella* cells. Results are also presented proving that the product of the *HUP1* gene is localized in the plasmalemma of D-Glc-induced *Chlorella* cells and is absent in plasma membranes of noninduced cells. Incubation of thin sections of *Chlorella* cells with anti-HUP1 antibodies and a fluorescence-labeled, second antibody yielded a ring of fluorescence on the surface of Glc-induced *Chlorella* cells.

The unicellular green alga *Chlorella kessleri* undergoes a metabolic switch from autotrophic to heterotrophic growth when D-Glc or certain other monosaccharides are present in the growth medium (Tanner, 1969; Tanner et al., 1970). These monosaccharides trigger the transcription of mRNAs coding for proteins such as mitochondrial and plasma membrane transporters and glycolytic enzymes. Differential screening of a cDNA library constructed from mRNA isolated from Glc-induced *Chlorella* cells allowed the isolation and identification of several cDNAs corresponding to these newly synthesized mRNAs (Sauer and Tanner, 1989; Hilgarth et al., 1991).

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The transport activity catalyzing the uptake of D-Glc, several other monosaccharides, or nonmetabolizable analogs of these sugars is induced more than 100-fold from very low basal levels (Komor and Tanner, 1971). In the presence of a membrane potential and/or a proton gradient, this transporter drives the accumulative transport of nonmetabolizable substrates such as 3-*O*-methylglucose across the plasmalemma of induced *Chlorella* cells and is therefore an energy-dependent monosaccharide-H^+ symporter (Komor and Tanner, 1976; Schwab and Komor, 1978). Sauer and Tanner (1989) reported on the cloning of a cDNA encoding a putative Glc-H^+ symporter from *Chlorella* that was named HUP1 for hexose uptake. This protein belongs to a large superfamily of membrane transporters (Marger and Saier, 1993) and its postulated function was later confirmed by expression of a full-length cDNA of HUP1 in *Schizosaccharomyces pombe* (Sauer et al., 1990a). Using this expression system it was shown that the kinetic parameters determined for the HUP1 protein expressed in *S. pombe* were almost identical to those determined for the inducible transporter in *Chlorella*. Results obtained from the expression of HUP1 in *Xenopus* oocytes (Aoshima et al., 1993) also suggested that the HUP1 protein might be identical with the inducible transport activity characterized in the plasma membranes of Glc-induced *Chlorella* cells.

There were, however, minor differences between the substrate specificity of the heterologously expressed HUP1 protein and the induced transport activity in *Chlorella*. Uptake of D-Gal by HUP1 was very slow in transgenic *S. pombe* and almost undetectable in *Xenopus* oocytes (Aoshima et al., 1993). In contrast, D-Gal is a good substrate for induced *C. kessleri* cells (Tanner et al., 1970). We were now able to identify two other cDNAs and genes from *C. kessleri*, *HUP2* and *HUP3*, which are highly homologous to the *HUP1* Glc-H^+ symporter gene. Expression of both genes in *Chlorella* is stimulated upon addition of Glc to the medium. This identification of two additional transcribed genes in *Chlorella* encoding putative hexose transporters makes it necessary to reconsider the possibility that HUP1 might not be the only sugar transporter of the *Chlorella* plasma membrane. In addition, there exists the possibility, although unlikely, that the localization of HUP1 protein in the plasma membranes of transgenic *S. pombe* cells is due to missorting of the protein in yeast cells and that HUP1 might also be localized in a different, perhaps intracellular membrane of *C. kessleri*. Recently, Villalba et al. (1992)

reported functional expression of a plant plasma membrane ATPase in yeast. In this case the protein was not found in the yeast plasma membrane but in the ER.

This paper reports on the cloning of *HUP2* and *HUP3* genes and cDNAs and on the induction of *HUP2* and *HUP3* mRNA transcription upon the addition of Glc. It is shown that the HUP2 and HUP3 proteins can be expressed functionally in *S. pombe* and that these proteins have different substrate specificities. Moreover, it is proven that the HUP1 protein is in fact localized in the plasma membrane of induced *Chlorella* cells and that it cannot be found in the membranes of noninduced cells. These data were obtained using an anti-HUP1 antibody that was raised against a fusion of β-galactosidase and the C terminus of the HUP1 protein.

MATERIALS AND METHODS

Strains

The strain of *Chlorella kessleri*, the growth conditions, and the induction of heterotrophic metabolism by D-Glc were the same as those described previously (Sauer and Tanner, 1984). For all cloning steps in *Escherichia coli*, the strain DH5α was used (Hanahan, 1983). The fission yeast strains used for expression of the *Chlorella* transporters were *Schizosaccharomyces pombe* strain *leu*1–32 (Beach and Nurse, 1981) and strain YGS-B25 (Milbrandt and Höfer, 1994). Strain YGS-B25 has no inherent plasma membrane monosaccharide transport activity. The strain TCY104 (strain YGS-B25 transformed with the yeast expression vector pEVP11) was generously provided by Dr. Thomas Caspari (University of Regensburg, Germany), and strain TCY96 (strain YGS-B25 expressing HUP1) has been published (Caspari et al., 1994). The *S. pombe* strains TCY12 (strain *leu*1–32 expressing HUP1) and TCY15 (strain *leu*1–32 transformed with the expression vector pEVP11) were described previously (Sauer et al., 1990a).

Cloning of *HUP2* cDNA and Genomic Clones

The λgt10 cDNA library of *Chlorella* used for differential screening was the same as that used previously (Sauer and Tanner, 1989). During the screening described in this paper a strongly induced cDNA was isolated (pTF9) that had no open reading frame. Rescreening of the library with the pTF9 insert resulted in the cloning of full-length cDNAs pTF352 and pTF354, which were highly similar to *HUP1* cDNAs. Conditions used for differential screening and for rescreening of the library were essentially the same as that described by Sauer and Tanner (1989).

^{32}P-labeled insert DNA of pTF9 was also used for the screening of a genomic library of *Chlorella* constructed in λgt10 (Wolf et al., 1991). One λ clone with a 5-kb insert was isolated (pTS1). This clone is lacking the 5′ end of the *HUP2* gene. Rescreening of the library with a 250-bp *Eco*RI/*Pst*I fragment from the 5′ end of pTF352 resulted in the isolation of a λ phage with a 4.5-kb insert composed of a 1500-bp *Eco*RI fragment (pLM601) and a 3-kb *Eco*RI fragment (pLM602). pLM601 contained the missing 5′ end of the *HUP2* gene. Conditions for screening of the genomic library were as described (Wolf et al., 1991).

Cloning of *HUP3* cDNA and Genomic Clones

The genomic library of *C. kessleri* was the same as that used previously (Wolf et al., 1991). During screening of this library with the ^{32}P-labeled insert of pTF14 (=truncated cDNA of *HUP1*; Sauer and Tanner [1989]) to obtain a HUP1 genomic clone, the clone pLM17 was isolated that had high similarity to HUP1 and HUP2 but was clearly different from these two genes. Due to this sequence homology the encoded gene was given the name *HUP3*. Clone pLM17 did not cover the entire *HUP3* gene and rescreening of the library with a 451-bp *Eco*RI/*Pst*I fragment from the 3′ end of pLM17 resulted in three positive clones: pLM18 (3613 bp), pLM28 (3927 bp), and pLM5 (about 6300 bp). pLM5 overlapped with the 3′ end of pLM17, and both clones together covered the complete sequence of the *HUP3* gene. Conditions for screening of the genomic library were as described (Wolf et al., 1991).

A full-length cDNA of HUP3 was obtained by running a PCR reaction with reverse-transcribed mRNA from Glc-treated *Chlorella* cells (Sauer and Tanner, 1984). The primers were made according to the putative 5′ and 3′ ends (HUP3–5 and HUP3–3, respectively; Table I) of the HUP3 genomic sequence. Both oligonucleotides were designed with internal *Eco*RI sites to allow direct cloning of the *Eco*RI-digested PCR product into the *Eco*RI site of pUC19. The resulting plasmid was named pTF314.

Nucleic Acid Sequencing

The full-length cDNA clone pTF352 (HUP2), the PCR-generated plasmid pTF314 (HUP3), and the modified 5′ and 3′ ends of HUP2PCR and HUP3PCR were sequenced (Sanger et al., 1977) using exonuclease III deletions or clone-specific sequencing primers.

RNA Isolation and Separation

Total RNA from *C. kessleri* was isolated and separated on agarose gels as described (Sauer and Tanner, 1989).

Table I. *Oligonucleotides used for isolation of HUP3 and for the expression of HUP2 and HUP3 in S. pombe*

Oligonucleotides used for amplification of HUP2 and HUP3. Restriction sites that were included into the oligonucleotide sequences are given in lowercase letters. ATG start and TAA stop codons are underlined.

HUP3–5: (26-mer)	5′-TTCAgaattcCCGCGGTAGCATACGA-3′
HUP3–3: (26-mer)	5′-CAGTgaattcAGGCGTTGTTTACTTC-3′
HUP21–5: (41-mer)	5′-AACTaagcttGTAAAAGAAATGGCAGGGGG CGGACCAGTAG-3′
HUP22–3: (18-mer)	5′-CTTGGGGACAGACAGCCC-3′
HUP31–5: (41-mer)	5′-AACTaagcttGTAAAAGAAATGGCCGGGGG CGCAATAGTAG-3′
HUP31–3: (55-mer)	5′-GGTGaagcttAGTGGTGGTGGTGGTGGTGC ctcgagTTCATGGTCTGAGAGATCC-3′

Expression of HUP2 and HUP3 in *S. pombe*

The 65 bp of 5′ untranslated sequence of the full-length cDNA clone pTF352 (encoding the HUP2 protein) were replaced by the 5′ untranslated sequence of the *Arabidopsis thaliana* STP1 monosaccharide-H^+ symporter clone pTF414RS11 (5′-AAGCTTGTAAAAGAAATG-3′; Sauer et al. [1990b]) in a single PCR reaction using the synthetic oligonucleotides HUP21–5 and HUP22–3 (Table I). The resulting DNA fragment had a *Hin*dIII site 14 bp upstream of the HUP2 start ATG (introduced during PCR) and a second *Hin*dIII site in the TAA stop codon (already present in the HUP2 sequence). After digestion with *Hin*dIII the resulting 1624-bp fragment was cloned into the yeast expression vector pEVP11 (Russel and Nurse, 1986) giving the plasmid pEVP::HUP2PCR, which was transformed into *S. pombe* strain YGS-B25. The transformed strain was named NSY25/2.

The 5′ untranslated sequence of HUP3 was also replaced by the 5′ untranslated sequence of the *A. thaliana* STP1 monosaccharide-H^+ symporter clone pTF414RS11 (Sauer et al., 1990b) in a single PCR reaction using *Chlorella* reverse-transcribed poly(A^+) RNA and the synthetic oligonucleotides HUP31–5 and HUP31–3 (Table I). The oligonucleotide HUP31–3 was designed in such a way that the resulting HUP3 C terminus was changed simultaneously from SQTMK* to SQTMNSRHHHHHH*, which is due to the introduction of a *Xho*I site (CTCGAG) and six CAC codons for His. The resulting DNA fragment had a *Hin*dIII site 14 bp upstream of the HUP3 start ATG (introduced during PCR) and a second *Hin*dIII site in the newly generated TAA stop codon. After digestion with *Hin*dIII the resulting 1642-bp fragment was cloned into the yeast expression vector pEVP11 giving the plasmid pEVP::HUP3PCR, which was transformed into *S. pombe* strain YGS-B25. The transformed strain was named NSY25/3.

Transport Tests and Determination of K_m Values

Transport of sugars into *S. pombe* cells was measured as described with 100 mM ethanol being present in all experiments (Sauer et al., 1990a, 1990b). For the determination of the K_m value of HUP1 in *S. pombe* strain YGS-B25, we used the strain TCY96 (Caspari et al., 1994). The pH was 5.0 for all measurements. All transport experiments were performed at least twice.

Purification of Anti-HUP1-A Antibodies

Anti-HUP1-A antiserum (AH1A antiserum) was raised in rabbits against a fusion protein of β-galactosidase and the 186 C-terminal amino acids of the HUP1 protein as previously described (Caspari et al., 1994). Antiserum was affinity purified (Olmsted, 1981) using extracts from transgenic *S. pombe* TCY12 cells expressing the HUP1 protein in the plasma membrane (Sauer et al., 1990b). SDS extracts from TCY12 total membranes were separated on polyacrylamide gels (Laemmli and King, 1970) and transferred to nitrocellulose filters (Dunn, 1986). The position of the HUP1 protein (42-kD apparent molecular mass) was determined by staining the lanes containing molecular mass markers. The respective part of the blot was excised and incubated with AH1A antiserum (3 mL of 1:20-diluted serum) for adsorption overnight at 4°C.

Bound antibodies were obtained from the filters as follows. Filters were washed five times for 2 min with blocking buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% skim milk powder, 0.1% Triton X-100) and rinsed with distilled water to remove excess serum. During an incubation in 0.5 mL of Gly buffer 2.8 (5 mM Gly, pH 2.8, 500 mM NaCl), most of the bound antibodies were released from the filters. After 2.5 min of incubation under continuous shaking the Gly buffer was immediately neutralized with 50 μL of 1 M Tris-HCl, pH 8.5, and put on ice after addition of 1 μL of 10% BSA. Residual antibodies were released in a second elution with Gly buffer 2.2 (5 mM Gly, pH 2.2, 500 mM NaCl). Both eluates were combined and concentrated with a Centricon 30 microconcentrator (Amicon, Beverly, MA) to the initial volume of the serum used for purification.

Preparation of Total Membranes from *S. pombe* and *C. kessleri*

Glc-induced and uninduced control cells of *C. kessleri* (Sauer and Tanner, 1984) were disintegrated with a French press (20,000 p.s.i.). Cell walls and unbroken cells were removed by low-speed centrifugation (5 min at 1,200*g* in a SS34 rotor) and total membranes were collected by spinning the supernatant for 30 min at 50,000*g* (SS34). The membrane pellet was resuspended in 1 mL of 1× SDS sample buffer (Laemmli and King, 1970).

Yeast cells were grown to an A_{578} of about 1 and total membranes were prepared as described (Caspari et al., 1994).

SDS-PAGE and Western Blot Analysis

SDS-polyacrylamide gels were prepared according to the protocol of Laemmli and King (1970), and western blots were performed according to Dunn (1986). Antibody binding was detected with the chemiluminescence western blot detection kit from Amersham, according to the manufacturer's protocol.

Preparation of Thin Sections of *Chlorella* Cells and *S. pombe*

Chlorella cells were induced or not induced for Glc transport as described (Sauer and Tanner, 1984), washed with water, and pelleted. *S. pombe* strains TCY12 (expressing HUP1) and TCY15 (control cells) were grown on minimal medium, harvested at an A_{578} of about 1, washed with water, and collected by centrifugation. Pellets (25 μL of packed cells each) were washed with buffer M (50 mM Mops, 5 mM EGTA, 2 mM $MgSO_4$, pH 6.4) and fixed in buffer M containing 0.1% glutaraldehyde and 6% formaldehyde for 1 h at 0°C. After three washes with buffer M (incubation times 2 min, 5 min, and 10 min) at 0°C the water was removed during three 10-min incubations in 30, 50, and 70% ethanol followed by two 15-min incubations in 100% ethanol (all at 0°C). Cells were embedded into methacrylate (Baskin et al., 1992) during an overnight in-

cubation in a 1:1 mixture of ethanol and a methacrylate mix (75% [v/v] butylmethacrylate, 25% [v/v] methylmethacrylate, 0.5% benzoine ethylether, 10 mM DTT), followed by two 4-h incubations in 100% methacrylate mix (all at 4°C). The material polymerizes during a final overnight incubation on ice under UV light (365 nm) in 100% methacrylate mix. Semithin sections (2 μm) were made with an ultramicrotome (Reichert, Wien, Austria), and sections were put on poly-L-Lys (Sigma, Deisenhofen, Germany) -coated coverslips.

Incubation of *C. kessleri* and *S. pombe* Thin Sections in Purified Antibodies

For removal of methacrylate from the thin sections, coverslips were incubated for 30 s in 100% acetone, washed three times with blocking buffer (without Triton X-100; see purification of anti-HUP1-A antibodies), and incubated for 30 min in blocking buffer without Triton and for 30 min in blocking buffer. After an overnight incubation with purified AH1A antibodies (diluted 1:2 in blocking buffer) the coverslips were washed three times with blocking buffer followed by a 1-h incubation with anti-rabbit IgG-fluoresceine isothiocyanate isomer I conjugate (1:300 diluted in blocking buffer; Sigma). After three final washes with blocking buffer, coverslips were rinsed with 100% methanol and mounted in FITC-Guard (Testoc, Inc., Chicago, IL). Photos were taken on a fluorescence phase microscope (Zeiss Standard 16) with an excitation light of 450 to 490 nm.

RESULTS

Cloning of *HUP2* Genomic and cDNA Sequences

The *Chlorella* HUP1 monosaccharide-H^+ symporter had been cloned by the differential screening of a λgt10 cDNA library constructed from mRNA of D-Glc-treated *Chlorella* cells with radiolabeled cDNAs from induced and noninduced algae (Sauer and Tanner, 1989). During this screening a truncated cDNA clone was isolated (pTF9) that showed some homology to *HUP1* sequences. Rescreening of the library with the pTF9 insert yielded two full-length clones (pTF352 with 2365 bp and pTF354 with 2384 bp) encoding a monosaccharide transporter-like protein. The longest open reading frame of these clones was 1620 bp yielding a protein with 540 amino acids and a calculated molecular mass of 58.28 kD. In Figure 1 the translated sequence of HUP2 is compared with the translated sequence of HUP1; similarities and homologies between these proteins are given in Table II.

Cloning of *HUP3* Genomic and cDNA Sequences

During the search for a genomic clone of the *Chlorella HUP1* monosaccharide-H^+ symporter in a genomic λgt10 library (Wolf et al., 1991), two clones were isolated that showed homology to the *HUP1* and *HUP2* genes (pLM5 with about 6300 bp and pLM17 with 4743 bp). The two clones covered the complete sequence of a yet unknown third monosaccharide transporter-like gene (named

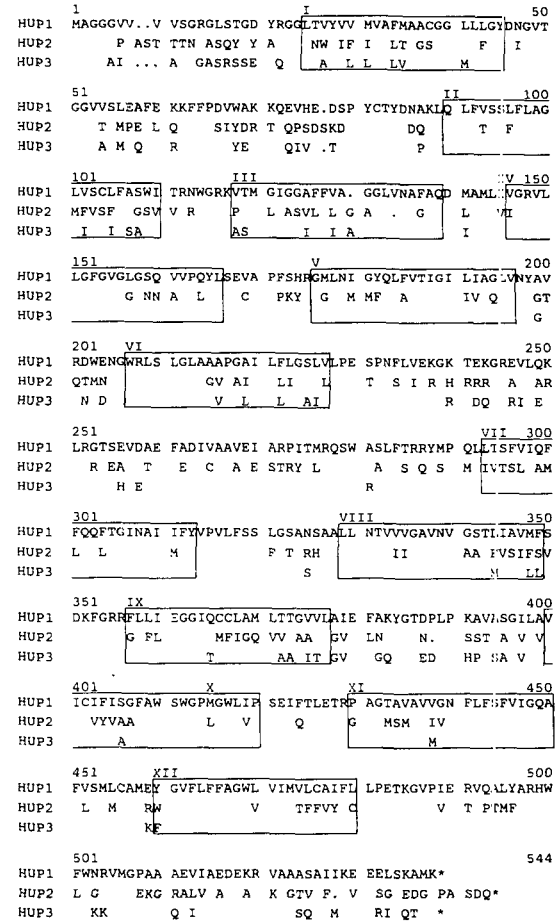

Figure 1. Sequence comparison of the translated sequences of the *C. kessleri* monosaccharide-H^+ symporters HUP1, HUP2, and HUP3. Only those amino acids of HUP2 and HUP3 that differ from the HUP1 sequence are shown. Dots represent gaps of one or more amino acid residues. Putative transmembrane helices are boxed and numbered with Roman numerals.

HUP3). Since the intron/exon border sequences of *Chlorella* are highly conserved (Wolf et al., 1991), a putative open reading frame of 1602 bp was predicted from this genomic sequence, encoding a putative protein of 534 amino acids and a calculated molecular mass of 57.77 kD (Table II).

To determine whether the *HUP3* gene is transcribed and to verify the putative open reading frame of the *HUP3* gene, the *Chlorella* cDNA library (see above) was screened with a 3′-specific probe from the putative 3′ untranslated sequence of the *HUP3* gene. The fragment used for hybridization started at an *Hpa*I site 303 bp downstream of the TAA stop codon and ended at a *Pvu*I site 736 bp further downstream. This region should be within the 3′ untranslated sequence of the *HUP3* gene for two reasons: first, the 3′ untranslated ends of all other *Chlorella* genes described to date are between 600 and 700 bp long (Hilgarth et al., 1991; Wolf et al., 1991), and second, a unique TGTAA algal polyadenylation signal was found 769 bp downstream of the TAA stop codon. This sequence is found in all *Chlorella* genes about 20 bp upstream of the polyadenylation site

Table II. *Comparison of the three HUP gene products from Chlorella*

Sequences of the three HUP gene products were analyzed and compared with the programs PEPTIDESORT and BESTFIT (gap weight: 3.0; gap length weight: 0.1) of the University of Wisconsin Genetics Computer Group. Percent similarity is shown on the lower left side; percent identity is given on the upper right side.

Parameter	HUP1	HUP2	HUP3
Amino acids	534	540	534
pI	7.5	9.6	8.4
Mol wt	57.59	58.28	57.77
	Percent identical amino acids		
	HUP1	HUP2	HUP3
Percent similar amino acids			
HUP1		56.5	83.1
HUP2	74.1		57.3
HUP3	91.7	75.7	

(Wolf et al., 1991). Screening of more than 150,000 plaque-forming units gave no positive signal, suggesting that the *HUP3* gene might not be transcribed in Glc-induced *Chlorella* cells. Screening of the same number of plaques with *HUP1* or *HUP2* probes yielded about 50 or 300 positive clones, respectively.

Using synthetic oligonucleotides (HUP3–5 and HUP3–3; Table I), which were designed to hybridize to sequences upstream from the ATG start codon und downstream of the TAA stop codon, it was finally possible to isolate a full-length cDNA of *HUP3* by PCR of mRNA from D-Glc-treated *Chlorella* cells. Sequencing of this PCR clone (pTF314) confirmed the open reading frame predicted from the genomic sequences. In Figure 1 the translated sequence of HUP3 is compared with the sequences of HUP1 and HUP2. The isolation of a *HUP3* cDNA by PCR also shows that the *HUP3* gene is transcribed in Glc-treated *Chlorella* cells. PCR of mRNA from noninduced *Chlorella* cells gave no detectable *HUP3* band.

Genomic Organization of the *Chlorella HUP3* Gene

The *HUP3* gene has 14 introns that are located at exactly the same positions as the 14 introns of the *HUP1* gene (Wolf et al., 1991). During the search for a genomic clone for the putative *HUP3* promoter the clone pLM503 was isolated. This clone was about 4000 bp long and sequences from the two ends revealed that pLM503 overlaps with the 3′ end of the *HUP1* gene on one end and with the 5′ end of the *HUP3* gene on the other end. Further sequencing showed that the TAA stop codon of the *HUP1* gene and the ATG start of the *HUP3* gene are separated by 2033 bp (Fig. 2). Information on the genomic organization of the *HUP2* gene is not available at the moment.

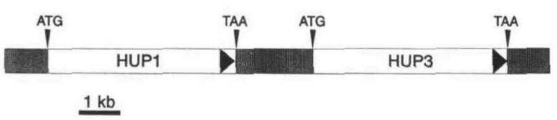

Figure 2. Genomic organization of the *Chlorella HUP1* and *HUP3* genes. The translation start (ATG) and stop sites (TAA) are indicated.

Induction of *HUP2* and *HUP3* mRNAs in D-Glc-Treated *Chlorella* Cells

HUP3 cDNA could be PCR amplified only from mRNA of *Chlorella* cells induced with D-Glc. Northern blot analyses were used for a better resolution of the induction kinetics of *HUP3* and to see whether *HUP2* is also induced by D-Glc. Total RNA was isolated at different times after addition of D-Glc to freshly harvested *Chlorella* cells and probed with 3′-specific probes of *HUP1*, *HUP2*, *HUP3*, and the control clone pTF30 (Sauer and Tanner, 1989). All three *HUP* genes are clearly induced by D-Glc (Fig. 3). The incubation times of radiolabeled northern blots on X-ray films, however, were about 10 times longer for *HUP3* than for *HUP1* and *HUP2*. This confirms the previous result (see above) indicating that *HUP3* is expressed to much lower levels than the other *HUP* genes. The induction of all *HUP* genes is extremely fast, with the first mRNA signals being detected as soon as 5 min after addition of D-Glc to the cells. In all three cases the mRNA levels reach a maximum after 10 to 30 min and decreased to a lower, constant level after that. The maximum of *HUP2* expression is reached later and seems to be sharper than that of the other *HUP* genes.

Expression of HUP2 and HUP3 in *S. pombe*

To study the function of the two additional transporters from *Chlorella*, we tried expression in *S. pombe*, a system that had proven to be very powerful for the functional expression of lower and higher plant transporters (Sauer et al., 1990a, 1990b). A *S. pombe* mutant (YGS-B25) deficient in Glc uptake (Milbrandt and Höfer, 1994) has been used to

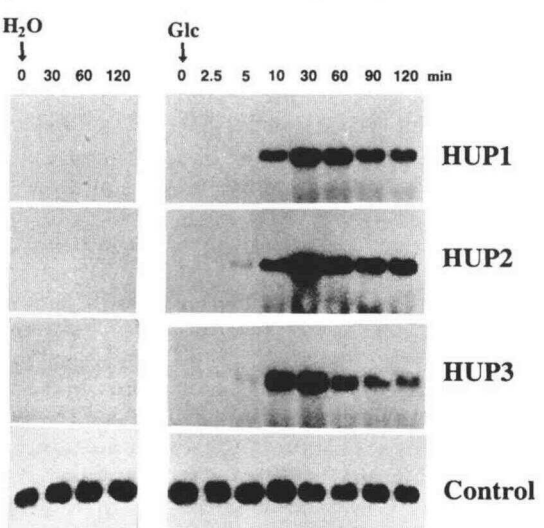

Figure 3. Northern blot analyses of the expression of *HUP1*, *HUP2*, and *HUP3* mRNAs in *C. kessleri*. RNA was isolated from *Chlorella* cells at the indicated times after addition of H_2O (control) or D-Glc (Glc) to the cells. Forty micrograms of total RNA were loaded per lane. Blots were probed with 3′-specific fragments for *HUP1*, *HUP2*, and *HUP3* and with the control cDNA pTF30 (Sauer and Tanner, 1989).

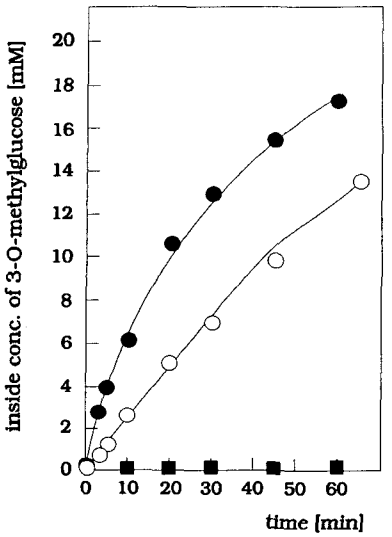

Figure 4. Transport of 3-*O*-methylglucose by *S. pombe* strains NSY25/2 (HUP2, ○), NSY25/3 (HUP3, ●), and TCY104 (control, ■). Uptake of ^{14}C-labeled 3-*O*-methylglucose was measured at a concentration of 100 μM at pH 5.0.

study structure/function relationships of mutations in the *Chlorella* HUP1 monosaccharide-H^+ symporter, and this mutant was able to accumulate 3-*O*-methylglucose more than 100-fold after transformation with the *Chlorella HUP1* cDNA under the control of the *S. pombe adh* promoter in the yeast expression vector pEVP11 (Caspari et al., 1994). At first we tried the expression of *HUP2* and *HUP3* by cloning the inserts of pTF352 (HUP2) or pTF314 (HUP3) into the unique *Hin*dIII site of pEVP11 and transformation into *S. pombe* YGS-B25. The transformants contained large amounts of *HUP2* or *HUP3* mRNA, but no additional transport activity could be detected (data not shown). This could be due to proteolytic degradation of the HUP2 and HUP3 proteins in the different transformants or to 5′ untranslated sequences in the *HUP2* and *HUP3* cDNAs that are not accepted by the *S. pombe* translation machinery.

To test the second possibility, the 5′ untranslated sequences of both cDNAs were replaced with the short 5′ untranslated sequence of the *A. thaliana* STP1 transporter by PCR. This sequence (5′-AAGCTTGTAAAAGAA-3′; Sauer et al. [1990b]) had an internal *Hin*dIII site for the cloning into pEVP11 followed by only 9 bases (8 purines and 1 pyrimidine). An inhibiting role of possible 3′ untranslated sequences could be excluded, since in the case of pTF352 (HUP2) the 3′ *Hin*dIII site used for the cloning into pEVP11 was within the TAA stop codon. The 3′ oligonucleotide for the PCR of HUP3 (oligonucleotide HUP31–3; Table I) was designed in such a way that an additional *Xho*I site plus six His residues were added in frame to the 3′ end of the *HUP3* cDNA followed by a synthetic *Hin*dIII cloning site within the TAA stop codon. It has been shown only recently that a very similar C-terminal modification of the *A. thaliana* STP1 monosaccharide-H^+ symporter had no influence on the substrate specificity and kinetic characteristics of the STP1 protein and that the His tag is very useful for the purification of the protein (Stolz et al., 1994).

Figure 4 shows that *S. pombe* YGS-B25 cells expressing *HUP2* (strain NSY25/2) or *HUP3* (strain NSY25/3) accumulate 3-*O*-methylglucose inside the cells (170- and 210-fold, respectively, after 60 min) when appropriate 5′ untranslated sequences were used. No sugar is transported by the control strain TCY104, which is transformed with the empty shuttle vector. In Table III the initial transport rates (first 3 min after addition of substrate) for different sugars are compared. The rates for D-Glc transport were set to 100%. It is obvious that the HUP3 protein has the same substrate preference as HUP1 (Table III). This is different for HUP2, which transports D-Gal better than D-Glc. On the other hand, Gal transport by HUP1 and HUP3 is only poor, and Fru, which is the second best sugar for HUP1 and HUP3, is not transported by HUP2.

We determined the K_m values for 3-*O*-methylglucose and D-Gal in NSY25/2 and NSY25/3 (Table IV) and compared them to values determined for HUP1 in strain TCY96. The close relationship of HUP1 and HUP3 is also obvious from these results. The K_m values of HUP1 and HUP3 are in the same order of magnitude, but those of HUP2 are about 50-fold lower.

Immunochemical Detection of HUP1 in Plasma Membranes of Induced *Chlorella* and Transgenic *S. pombe*

To prove that plant monosaccharide transporters, which are found in the plasma membranes of heterologously transformed cells expressing hexose symporters (Sauer et al., 1990a, 1990b; Boorer et al., 1992; Aoshima et al., 1993; Sauer and Stadler, 1993), are also localized on the plasma membrane of plant cells, we tried to immunolocalize the *Chlorella* HUP1 transporter in *Chlorella* plasma membranes. The advantage of this system is the inducibility of the monosaccharide transport in these cells and the existence of both induced cells expressing and control cells not expressing the transporter.

Crude antiserum against a β-galactosidase/HUP1 fusion protein identifies the HUP1 protein on western blots of SDS extracts of both Glc-treated *Chlorella* cells (Fig. 5A) and transgenic *S. pombe* (Fig. 5B). Affinity purification of this

Table III. *Comparison of the initial transport rates of the Chlorella HUP transporters expressed in S. pombe*

Transport rates for seven different monosaccharides (all of 100 μM) were determined in transgenic yeast cells in the presence of 100 mM ethanol over the first 3 min after substrate addition. One hundred percent transport rates for D-Glu by HUP1, HUP2, and HUP3 were 170, 13, and 185 μmol h^{-1} g^{-1} fresh weight, respectively (average of at least three experiments).

Monosaccharide	HUP1	HUP2	HUP3
D-Glu	100	100	100
D-Fru	82	0	77
D-Gal	8	141	5
D-Man	55	32	54
D-Xyl	15	100	12
3-*O*-Methylglucose	10	98	12
L-Glu	<1	3	<1

Table IV. *Apparent K_m values of the HUP1, HUP2, and HUP3 monosaccharide-H^+ symporters from Chlorella (mM)*

Values were determined in transgenic *S. pombe* YGS-B25 cells expressing the respective cDNA.

Monosaccharide	HUP1	HUP2	HUP3
3-*O*-Methylglucose	1.0[a]	0.020	0.4
D-Gal	3.0	0.025	0.9

[a] This value was taken from Sauer et al. (1990a).

serum with recombinant HUP1 protein purified from transgenic yeast cells causes a further increase in specificity, and there is no residual cross-reaction of the AH1A antiserum with any other protein when tested in yeast cells (Fig. 5C). Both crude and purified antiserum bind to monomeric HUP1 protein (42 kD) and to various higher molecular mass aggregates of HUP1. Only the affinity-purified AH1A antiserum was used for the immunolocalization of HUP1 in cross-sections of *Chlorella* and the transgenic yeast strain TCY12. Figure 6 shows that only thin sections of induced *Chlorella* cells (A) yield a yellowish green fluorescence signal on the cell surface; sections from noninduced *Chlorella* cells (B) show no fluorescence at all. In Figure 7, similar thin sections from transgenic *S. pombe* cells TCY12 (HUP1 expressing; A) and TCY15 (control; B) are shown. This latter result demonstrates that the majority of the HUP1 protein in *Chlorella* and of the recombinant HUP1 protein in *S. pombe* is found in the plasma membranes of these cells. No protein is detectable with this assay in internal membranes such as the ER, which has been described for the plant plasma membrane ATPase (Villalba et al., 1992).

DISCUSSION

The data presented in this paper show that *C. kessleri* possesses a family of three HUP genes. One of them, HUP1, had previously been shown to catalyze Glc-H^+ symport into transgenic cells of *S. pombe* (Sauer et al., 1990a) and into a vesicular in vitro system (Opekarová et al., 1994).

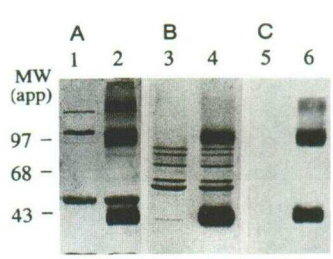

Figure 5. Western blot detection of HUP1 protein in total membranes of Glc-induced *C. kessleri* (lane 2; lane 1 is the uninduced control) or transgenic *S. pombe* strain TCY12 (lanes 4 and 6; lanes 3 and 5 are extracts from the *S. pombe* control strain TCY15). Blots A and B were treated with unpurified AH1A antiserum (diluted 1:250), and blot C was treated with affinity-purified AH1A antiserum (diluted 1:100). Identical amounts of protein (about 8 μg) were separated on each lane. The antiserum binds to monomeric (42 kD), dimeric (84 kD), and oligomeric HUP1 protein both in *Chlorella* and yeast. Apparent molecular masses of standards [MW(app)] are given in kD.

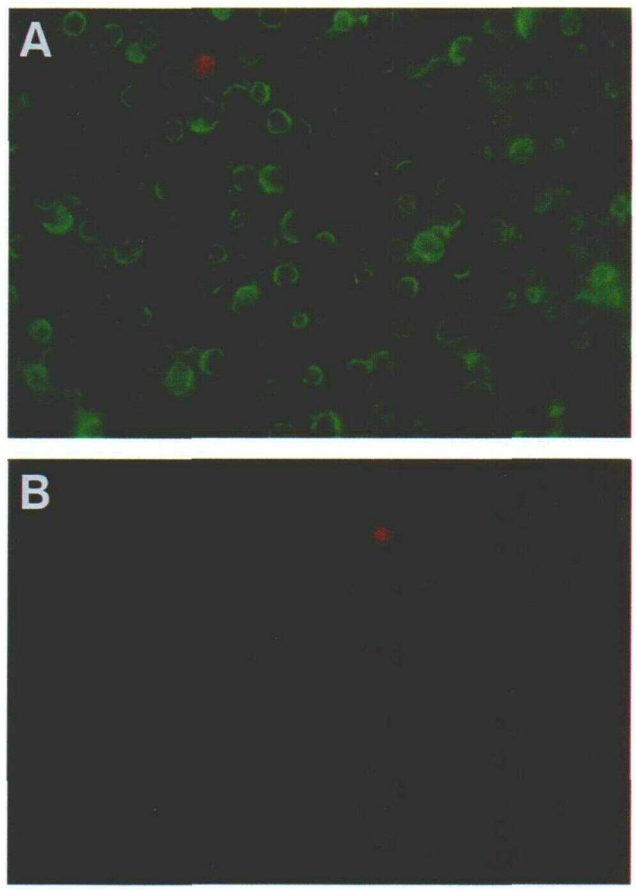

Figure 6. Thin sections of induced (A) and uninduced (B) *C. kessleri* cells treated with affinity-purified AH1A antiserum and FITC-coupled second antibody.

First attempts to express full-length cDNA clones of both *HUP2* or *HUP3* in *S. pombe* resulted in the transcription of the respective mRNAs in transformed yeast cells but with no measurable Glc transport activity (data not shown). This problem was overcome by replacing the 5′ untranslated sequences of *HUP2* and *HUP3* with those from the *A. thaliana* STP1 Glc-H^+ symporter, which was successfully expressed in *S. pombe* and which had a very short, pyrimidine-rich 5′ untranslated sequence. The products of the *HUP1* and *HUP3* genes are very similar not only in their primary sequence (Fig. 1; Table II) but also in their substrate specificities (Table III) and their apparent K_m values (Table IV). The additional observation that both genes are tandemly connected in the *Chlorella* genome suggest that *HUP1* and *HUP3* arose from a gene duplication in the recent evolutionary period. The *HUP2* gene, however, which differs in all these points from *HUP1* and *HUP3*, seems to be less closely related. This is also confirmed by comparison of the intron positions in the three different *HUP* genes; whereas all 14 introns of the *HUP1* and *HUP3* genes are at identical positions within the two genes, intron 4 is missing in the *HUP2* gene (data not shown).

PCR studies, northern blot analyses, and results from λgt10 cDNA library screening suggest that the level of *HUP3* mRNA reached after D-Glc induction is much lower

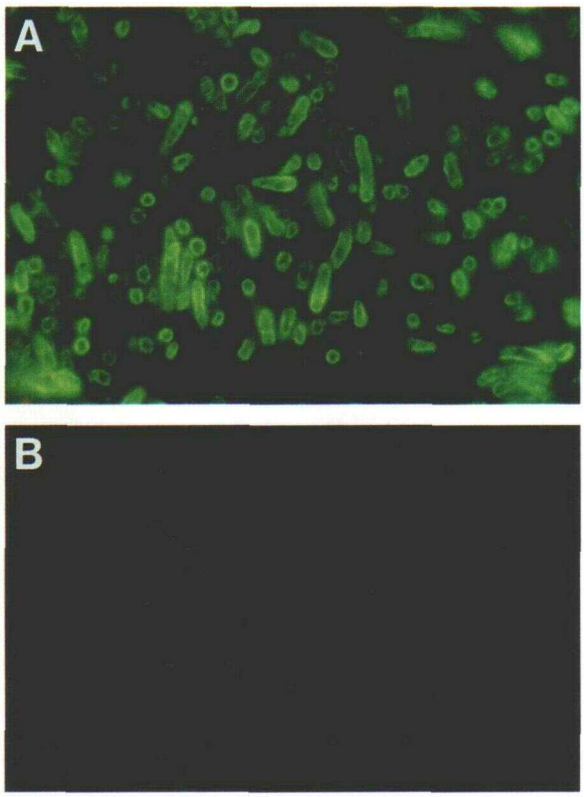

Figure 7. Thin sections of *S. pombe* strain TCY12 expressing HUP1 (A) and the control strain TCY15, which was transformed with the pEVP11 expression vector (B) (Sauer et al., 1990b), were treated with affinity-purified AH1A antiserum and FITC-coupled second antibody.

than the levels of *HUP1* and *HUP2* mRNAs. This indicates that the HUP3 protein possibly does not play a very important role for monosaccharide transport of induced *Chlorella* cells. The two other transporters, however, seem to be expressed to much higher levels.

The sequence comparisons shown in Figure 1 reveal regions of high conservation among all three HUP transporters and other regions where the similarity is much more pronounced between the Glc/Fru transporters HUP1 and HUP3. Such comparisons may allow researchers to distinguish regions of general importance for monosaccharide and/or proton transport and regions that are responsible for the recognition of different substrates such as D-Fru or D-Gal. Although D-Gal is normally not a good substrate of Glc-specific transporters, D-Glc seems to be well accepted even by Gal transporters that are normally repressed by D-Glc (Henderson et al., 1977). This is also the case for the *Chlorella* HUP1 and HUP2 transporters (Table III).

The identification of two highly induced H^+ symporters for Glc/Fru (HUP1) and Gal (HUP2) explains the differences that were seen when the activities of HUP1 in transgenic *S. pombe* and *Xenopus* were compared with the monosaccharide transport in induced *Chlorella* cells. In contrast to the good transport rates for D-Gal in *Chlorella* (Tanner et al., 1970), only poor transport rates were found in the heterologous expression systems (Table III; Aoshima et al., 1993). It should also be pointed out that the coinduction of Gal and Glc transporters by Glc is quite unusual; commonly, Glc represses Gal transport (Henderson et al., 1977; Bisson et al., 1993).

Until now the plasma membrane localization of cloned plant sugar transporters has never been directly shown. Rather it was extrapolated from kinetic similarities of expressed proteins and whole-cell measurements in the respective plant cell and from the plasma membrane localization in heterologous expression systems. The only possible way to prove unequivocally that proteins such as HUP1 or HUP2 are monosaccharide-H^+ symporters of the *Chlorella* plasmalemma is to show that these proteins are in fact localized in the plasma membrane. This is demonstrated by the data presented in Figure 6 of this paper, where fluorescence is seen only as a single ring corresponding to the cell surface of *Chlorella*. In addition, the fluorescence signal is obtained only with cells induced for Glc uptake.

Additional PCR studies on *Chlorella* mRNAs with oligonucleotides made against highly conserved sequences of *HUP1*, *HUP2*, and *HUP3* seem to indicate that there is no further closely related member of the *Chlorella HUP* family. Since HUP1, HUP2, and HUP3 are all induced by D-Glc and other monosaccharides, and since they are catalyzing the first step during heterotrophic growth of plant cells, the import of organic compounds, it will be of general interest to understand the involved induction pathway in the future.

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LITERATURE CITED

Aoshima H, Yamada M, Sauer N, Komor E, Schobert C (1993) Heterologous expression of the H^+/hexose cotransporter from *Chlorella* in *Xenopus* oocytes and its characterization with respect to sugar specificity, pH and membrane potential. J Plant Physiol **141:** 293–297

Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: localization of microtubules and other antigens throughout developing organs in plants of diverse taxa. Planta **187:** 405–413

Beach D, Nurse P (1981) High frequency transformation of the fission yeast *Schizosaccharomyces pombe*. Nature **290:** 140–142

Bisson LF, Coons DM, Kruckeberg AL, Lewis DA (1993) Yeast sugar transporters. Crit Rev Biochem Mol Biol **28:** 259–308

Boorer KJ, Forde BG, Leigh RA, Miller AJ (1992) Functional expression of a plant plasma membrane transporter in *Xenopus* oocytes. FEBS Lett **302:** 166–168

Caspari T, Stadler R, Sauer N, Tanner W (1994) Structure/function relationship of the *Chlorella* glucose/H^+-symporter. J Biol Chem **269:** 3498–3502

Dunn SD (1986) Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on western blots by monoclonal antibodies. Anal Biochem **157:** 144–153

Hanahan D (1983) Studies on transformation of *E. coli* with plasmids. J Mol Biol **166:** 577–580

Henderson PJF, Giddens RA, Jones-Mortimer MC (1977) Transport of galactose, glucose and their molecular analogues by *Escherichia coli* K12. Biochem J **162:** 309–320

Hilgarth C, Sauer N, Tanner W (1991) Glucose increases the expression of the ATP/ADP translocator and the glyceraldehyde-3-phosphate dehydrogenase genes in *Chlorella*. J Biol Chem **266:** 24044–24047

Komor E, Tanner W (1971) Characterization of the active hexose transport system of *Chlorella vulgaris*. Biochim Biophys Acta **241:** 170–179

Komor E, Tanner W (1976) The determination of the membrane potential of *Chlorella vulgaris*. Eur J Biochem **70:** 197–204

Laemmli UK, King J (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Marger MD, Saier MH (1993) A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. Trends Biol Sci **18:** 13–20

Milbrandt B, Höfer M (1994) Glucose-transport-deficient mutants of *Schizosaccharomyces pombe*: phenotype, genetics and use for genomic complementation. Microbiology **140:** 2617–2623

Olmsted JB (1981) Affinity purification of antibodies from diazotized paper blots of heterogenous protein samples. J Biol Chem **256:** 11955–11957

Opekarová M, Caspari T, Tanner W (1994) The *HUP1* gene product of *Chlorella kessleri*: H^+/glucose symport studied in vitro. Biochim Biophys Acta **194:** 149–154

Russel P, Nurse P (1986) $cdc25^+$ functions as an inducer in the mitotic control of fission yeast. Cell **45:** 145–153

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Sauer N, Caspari T, Klebl F, Tanner W (1990a) Functional expression of the *Chlorella* hexose transporter in *Schizosaccharomyces pombe*. Proc Natl Acad Sci USA **87:** 7949–7952

Sauer N, Friedländer K, Gräml-Wicke U (1990b) Primary structure, genomic organization and heterologous expression of a glucose transporter from *Arabidopsis thaliana*. EMBO J **9:** 3045–3050

Sauer N, Tanner W (1984) Partial purification and characterization of inducible transport proteins of *Chlorella*. Z Pflanzenphysiol **114:** 367–375

Sauer N, Tanner W (1989) The hexose carrier from *Chlorella*. cDNA cloning of a eucaryotic H^+-cotransporter. FEBS Lett **259:** 43–46

Schwab W, Komor E (1978) A possible mechanistic role of the membrane potential in proton-sugar cotransport of *Chlorella*. FEBS Lett **87:** 157–160

Stolz J, Stadler R, Opekarová M, Sauer N (1994) Functional reconstitution of the solubilized *Arabidopsis thaliana* STP1 monosaccharide-H^+ symporter in lipid vesicles and purification of the histidine tagged protein from transgenic *Saccharomyces cerevisiae*. Plant J **6:** 225–233

Tanner W (1969) Light-driven uptake of 3-O-methylglucose via an inducible hexose uptake system of *Chlorella*. Biochem Biophys Res Commun **36:** 278–283

Tanner W, Grünes R, Kandler O (1970) Spezifität und Turnover des induzierbaren Hexose-Aufnahmesystems von *Chlorella*. Z Pflanzenphysiol **58:** 376–386

Villalba JM, Palmgren MG, Berberian GE, Ferguson C, Serrano R (1992) Functional expression of plant plasma membrane H^+-ATPase in yeast endoplasmic reticulum. J Biol Chem **267:** 12341–12349

Wolf K, Tanner W, Sauer N (1991) The *Chlorella* H^+/hexose cotransporter gene. Curr Genet **19:** 215–219